Sequence variation in the cadherin gene of *Ostrinia nubilalis*: a tool for field monitoring

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Abstract

Toxin-binding proteins of insect midgut epithelial cells are associated with insect resistance to *Bacillus thuringiensis* (Bt) Cry toxins. A 5378 nt cDNA encoding a 1717 amino acid putative midgut cadherin-like glycoprotein and candidate Cry1Ab toxin-binding protein was characterized from *Ostrinia nubilalis*. Intraspecific alignment of partial *O. nubilalis* cadherin gene sequences identified variance within proposed Cry1A toxin binding region 2 (TBR2), ^{1328}IPLQTSILVVT[I/V] N^{1340}, and flanking Cry1A toxin binding region 1 (TBR1), ^{861}DIEIEIIDTNN^{871}. DNA sequence and PCR-RFLP detected single nucleotide polymorphism between cadherin alleles, and pedigree analysis demonstrated Mendelian inheritance. A population sample from Mead, Nebraska showed allelic polymorphism. These assays may be useful for linkage mapping and field surveillance of wild populations and of *O. nubilalis*. Published by Elsevier Ltd.

Keywords: Cadherin; *Bacillus thuringiensis* receptor; Midgut cDNA

1. Introduction

Resistance to insecticides commonly evolves in agricultural fields when insecticides are frequently applied at high doses to control insect pests (Georghiou and Lagunes-Tejeda, 1991). The western corn rootworm (*Diabrotica* sp.; Miota et al., 1998; Zhu et al., 2001) evolved field resistance to carbaryl insecticides. Indian mealmoth (*Plodia interpunctella*; McGaughey, 1985) and diamondback moth (*Plutella xylostela*; Tabashnik et al., 1990) evolved resistance to insecticides with toxins of the bacterium *Bacillus thuringiensis* var. kurstaki. Most of the documented instances of insect resistance involve a small number of genes (Roush and McKenzie, 1987). Insecticide resistance occurred by a single amino acid change in acetylcholinesterase, the target of organophosphate and carbamate insecticides in house fly (Williamson et al., 1996), and the sodium channel peptide targeted by pyrethroids in cockroach (Miyazaki et al., 1996). Similarly, cyclodiene resistance occurs by a single nonsynonymous point mutation in the *Drosophila* γ-aminobutyric acid (GABA) receptor (ffrench-Constant et al., 1993).

European corn borer, *Ostrinia nubilalis* (Lepidoptera Crambidae), a lepidopteran pest of maize, has been controlled with foliar applications of the bacterium *B. thuringiensis* (Bt) var. kurstaki. Incorporation of the *B. thuringiensis* Cry1Ab toxin gene into maize germplasm (Koziel et al., 1993) results in expression that controls *O. nubilalis* larvae (Walker et al., 2000). High selection pressure and adaptation to transgenic toxins by *O. nubilalis* larvae raises concerns regarding sustainability of Bt technology. Commercial hybrid maize expressing Cry1Ab toxins were grown on 29% of 2003 United States corn acres (22.9 million acres; USDA-NASS, 2003). Resistance has not been detected in the field; but

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moderate resistance was found in *O. nubilalis* laboratory colonies fed artificial Bt containing diets (Bolin et al., 1999; Huang et al., 1999; Chaufaux et al., 2001).

Modification of midgut receptor proteins that bind Cry toxins may reduce susceptibility of insects. Ligand blots demonstrate that Cry1Ab binds to a 220-kDa cadherin-like glycoprotein, and 145 and 154-kDa aminopeptidase (APN) isoforms in *O. nubilalis* (Hua et al., 2001). A *Manduca sexta* 210-kDa E-cadherin-like peptide bound activated (trypsinized) Cry1A toxin (Francis and Bulla, 1997). Midgut expressed cadherin-like cDNAs were isolated from *M. sexta* (Vadlamudi et al., 1995), *Bombyx mori* (Nagamatsu et al., 1998a), *Heliothis virescens* (Gahan et al., 2001), and *Pectinophora gossypiella* (Morin et al., 2003). Furthermore, a cadherin knockout from a transposon insertion resulted in 40,000-fold resistance in the *H. virescens* YHD2 colony (Gahan et al., 2001). Segregation of alleles at a cadherin locus was associated with resistance in *P. gossypiella* strain AZP-R (Morin et al., 2003). CRY1A domain II binds lepidopteran midgut epithelial receptors (de Maagd et al., 2001) by interacting with two cadherin motifs (Gomez et al., 2003). Cadherin Cry1A toxin binding region (TBR1), [865]NITIHITDTNN[875], was identified on *M. sexta* gene Bt-R$_1$ and implicated in binding loop 2 of CRY1A domain II (Gomez et al., 2001, 2002a, b). A second binding region (TBR2) was identified in *B. mori* cadherin repeat 9 (CR9; Nagamatsu et al., 1999) and *M. sexta* cadherin CR11 (Dorsch et al., 2002). Cadherin TBR2 was narrowed to 12 amino acids of Bt-R$_1$, [1331]IPLPASILTVTV[1342], and predicted to interact with loop α-8 of CRY1A domain II (Gomez et al., 2003). Effects of cadherin toxin binding region variation on resistance traits are unknown.

The extracellular domain of cadherin peptide also has been implicated as a Cry1Ab binding receptor in the *O. nubilalis* midgut (Hua et al., 2001). Candidate Cry1Ab binding receptor genes have not been reported in *O. nubilalis*. In this paper, we report research that isolated a full-length midgut cadherin-like cDNA from 5th instar *O. nubilalis* larvae, determined allelic variation (single nucleotide polymorphism; SNPs) in and near two putative Cry1Ab toxin-binding regions (TBR1 and TBR2), and characterized Mendelian segregation of SNPs among pedigrees by PCR-RFLP. Additionally, in a preliminary field screening experiment we estimated cadherin allele variation in a susceptible population of *O. nubilalis* near Mead, Nebraska.

2. Materials and methods

2.1. Cadherin alignment and phylogeny

A 3048 residue consensus alignment was constructed among 11 lepidopteran cadherins, excluding *O. nubilalis*, using Align X software (Informax, San Francisco, CA; gap penalty = 5). Regions of peptide similarity were identified and corresponding cDNA sequence used to design degenerate 3′ RACE oligonucleotides with Primer3 (Rozen and Skaletsky, 1998; see primer Cad3pR in Section 2.3). Twelve lepidopteran cadherin amino acid sequences, including *O. nubilalis*, were placed into a single phylogeny using programs from the PHYLIP package (Felsenstein, 1989). One thousand bootstrap resampling steps were produced by the SeqBoot program, parsimony trees were generated using AAPars, a strict consensus tree was estimated from all possible phylogenies with CONSENSE, and was viewed using TreeView (Page, 1996).

2.2. Complementary DNA (cDNA) synthesis and cloning

Ten dissected larval *O. nubilalis* midguts were bulked, ground to powder in liquid nitrogen, and RNA extracted with RNeasy extraction kits (Qiagen, Valencia, CA) according to manufacturer instructions. First strand cDNA synthesis used 0.5 μM of poly(T) adapter (PT-AD; GGT GTA ATA CGA CGG CCT GGA ATT CTT TTT TTT TTT TTT TTT T), 2 μg of total RNA, 40 U AMV reverse transcriptase (Promega, Madison, WI) in a 20 μl reaction volume incubated at 42 °C for 1 h. Primer Cad3pR (TMA ACA ACA CCC CTC CYA CKC T) was designed using Primer3 (Rozen and Skaletsky, 1998) to anneal transcript sequence corresponding to a NNTPPLT amino acid sequence, which was conserved among lepidopteran cadherins. Hot start PCR of first strand cDNA used 5 pmol each of degenerate primer Cad3pR and poly(T) adapter primer 1 PT-Adc1 (GTG TAA TAC GAC GGC CTG G), 1.0 U *Tli* proofreading polymerase (Promega), 100 μM dNTPs, 1.5 mM MgCl$_2$, and 2.5 μl of 10 × thermal polymerase buffer (Promega) in a 25 μl reaction volume. A PTC-100 thermocycler (MJ Research, Watertown, MA) cycled 40 times at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 4 m. Amplification was confirmed and fragment size estimated by electrophoresis of 10 μl PCR product on a 10 cm 1.5% agarose 1X tris-borate EDTA gel containing 0.5 μg/ml ethidium bromide. PCR product was ligated using the pGEM-T easy cloning system (Promega) by overnight incubation at 4 °C, and was used to transform 80 μl *E. coli* SURE (Stratagene). DNA sequencing of plasmid inserts used dye-terminator cycle sequencing (DTCS) quick-start kits (Beckman-Coulter, Fullerton, CA) and fragments separated on a CEQ8000 capillary electrophoresis system (Beckman-Coulter). Identity of insert sequence was checked by BLAST search, and transcript-specific 5′ RACE primers designed (Primer3; Rozen and Skaletsky, 1998).

Oligonucleotide primer Cad5pR (CTG CAT CGA TCG CCT CGT T) was designed from 3′ RACE cDNA

sequence. First Choice RLM RACE kit (Ambion) was used to isolate 5′ cDNA end of the *O. nubilalis* cadherin transcript according to manufacturer instructions; RLM-RACE template was generated in 20 μl reactions with 1 μg of midgut total RNA. Subsequent hot start PCR used 1.0 U *Tli* proofreading polymerase (Promega), 200 μM dNTPs, 1.5 mM MgCl$_2$, and 2.5 μl of 10 × thermal polymerase buffer (Promega), 1 μl RLM mRNA template, 5 pmol each of degenerate primer Cad5pR, and 1 μl RLM-RACE 5′ adapter outer primer (Ambion; GCT GAT GGC GATG AAT GAA CAC) in a 25 μl reaction. A PTC-100 thermocycler (MJ Research) cycled 40 times at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 4 m. RT-PCR products were ligated, cloned, and sequenced as described previously.

2.3. Polymorphism with putative cadherin Cry1Ab toxin binding domains

Two putative Cry1A toxin-binding domains, [861]DIEIEIIDTNN[871] (TBR1) and [1328]IPLQT-SILVVTV[1339] (TBR2), were identified from a single 1717 residue *O. nubilalis* midgut cDNA open reading frame (ORF). TBR1 and TBR2 were PCR amplified using primers OnCadTBR1-F (CAA ATT GAG GTG GAT GCG AA) with OnCadTBR1-R (TGT CAC GGT CGT GGG AGT ACA), and OnCadTBR2-F (TYT CTT CAA CAG ACG GCA ACA A) with On-CadTBR2-R (ACC CTG ACG GTA AGC AAT TCC C). PCR products were referred to as *O. nubilalis* Cry1A toxin binding region 1 (OnTBR1) and 2 (OnTBR2), and amplified using 2.5 mM MgCl$_2$, 50 μM dNTPs, 7.5 pmol of each primer, 0.9 U *Taq* DNA polymerase (Promega), and 100 ng of DNA template (equal mix of 10 individual DNA samples) in a 12.5 μl reaction. PTC-100 thermocycler conditions used 95 °C for 3 m, followed by 40 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 20 s. OnTBR1 and OnTBR2 were ligated into pGEM-T vector (Promega), and used to transform *E. coli* SURE (Stratagene). Plasmid inserts were sequenced as described previously. Sequence data were imported into Vector NTI Suite 7.0 (Informax, San Francisco, CA) and multiple sequence alignments were made using AlignX software (Informax; gap penalty = 5).

A third PCR product (OnTBR2-s) used primer OnCadTBR2-F with OnCadTBR2-R2 (CTY AGC TTT TGC ASG AAG AAA G) to amplify an *O. nubilalis* cadherin gene region containing [1328]IPLQT-SILVVTV[1339]. The OnTBR2-s PCR product is smaller and eliminates length polymorphism contained within an intron spanned by the original OnTBR2 product, thus facilitated ease of SNP genotyping. Polymorphic restriction endonuclease cleavage sites from OnTBR1, *Nde*II, *Rsa*I, *Taq*I, and *Tsp*509I, and OnTBR2, *Cfo*I, *Hae*III, and *Hpy*CH4III, were identified from DNA sequence alignments. All endonucleases were purchased from Promega, except *Tsp*509I and *Hpy*CH4III (New England BioLabs). PCR-RFLP reactions of fragments OnTBR1 and OnTBR2-2 used PCR conditions described previously scaled to 25 μl. *Cfo*I, *Nde*II, *Rsa*I, *Taq*I, and *Tsp*509I digests included 6.0 μl of PCR product, 2.5 μl 10 × Buffer, 0.1 mg/μl BSA, and 0.25 U of enzyme in 25 μl. OnTBR2-2 *Hae*III and *Hpy*CH4III double digestions used 0.5 U of each enzyme. Reactions were incubated at 37 or 60 °C (*Tsp*509I) for 8–14 h. Entire digest volumes were loaded onto 1.0 mm × 16 cm 6% polyacrylamide (19:1 acrylamide: bisacrylamide) 0.5 × TBE gels, and separated at 140 V for 6 h. A 25 bp step-ladder (Promega) was used for size comparison. Gels were stained with ethidium bromide, and digital images taken under UV illumination on a PC-FOTO/Eclipse Documentation System (Fotodyne, Hartland, WI). Allele frequencies for cadherin loci were estimated from 96 *O. nubilalis* adults.

2.4. Cadherin allele frequencies and Mendelian inheritance

Over 300 adult *O. nubilalis* were collected in a single light trap at a non-Bt corn field near Mead, Nebraska in August 2002 (contributed by John Seymour, University of Nebraska), and were samples stored separately in 1.5 ml microcentrifuge tubes at −20 °C. A subsample of 92 individuals was screened with previously described PCR-RFLP protocols, and used to provide baseline estimates cadherin allele frequencies in a given population.

Two paired matings of *O. nubilalis* from the USDA-ARS, Corn Insects and Crop Genetics Research Unit laboratory colony were used to establish F$_1$ families. The F$_1$ progeny were sib-mated to produce F$_2$ larvae. F$_2$ larvae were reared to adults and used to estimate genotype frequencies for cadherin loci. Larvae were reared on artificial diet (Guthrie, 1987) and adult samples were stored separately in 1.5 ml microcentrifuge tubes at −20 °C prior to DNA isolation. DNA from field and pedigree samples was extracted from adult thorax tissue using methods described by Coates and Hellmich (2003). Genotyping by PCR-RFLP was performed as described previously. The goodness-of-fit of observed genotypic distributions during the F$_2$ generation were compared to expectations based on Mendelian inheritance.

3. Results and discussion

3.1. O. nubilalis cadherin identification

A 220-kDa cadherin-like peptide from *O. nubilalis* was identified as a midgut Cry1Ab receptor (Hua et al., 2001). We isolated a 5378 nt *O. nubilalis*

midgut-expressed mRNA (AY612336) similar to lepidopteran cadherin transcripts (*Helicoverpa armigera* AF519180, BLAST score = 92, E-value 10^{-14}; *B. mori* AB041509, BLAST score = 54, E-value 0.003). A single 5154 nt open reading frame (ORF) encoded a putative 1717 amino acid polypeptide (Fig. 1). Protein Database (PDB) search with PredictProtein (Rost, 1996) demonstrated high similarity (S) and identity (IDE) of the putative *O. nubilalis* peptide to *B. mori* BtR175 (S = 73%, IDE = 61%; Nagamatsu et al., 1998b), *L. dispar* BTR-CAD (S = 72%, IDE = 60%; AF317621), *M. sexta* Bt-R1 (S = 58%, IDE = 72%; Vadlamudi et al., 1995) and *H. virescens* BtR-4 (S = 58%, IDE = 72%; Gahan et al., 2001). A parsimony-based phylogeny used a consensus 3048 amino acid alignment of 12 lepidopteran cadherins including *O. nubilalis* and indicated three main divisions: cadherins from superfamilies Noctuoidae, Bombycoidea, Sphingoidea, and Pyraloidea and Gelchioidea (Fig. 2). Internal branch support at internal nodes is greater than 85% based on 1000 bootstraps, but terminal nodes among *B. mori* alleles may show homoplasy due to homologous recombination. Placement of the *O. nubilalis* ORF within the cadherin peptide phylogeny and

high peptide similarity to other lepidopteran cadherin, suggests the *O. nubilalis* midgut cDNA (AY612336) encodes a cadherin-like peptide.

Cadherin-like motifs were identified within the derived *O. nubilalis* polypeptide. A ProSite database search identified a 1402 amino acid long extracellular domain, a 21 residue signal peptide (SIG), transmembrane region (TMR), and cytoplasmic domain (CPD; Fig. 1). Eleven consensus cadherin repeat motifs ([LIV]-x-[LIV]-x-D-x-N-D-[NH]-x-P) were identified by PredictProtein (Rost, 1996). The 1717 amino acid *O. nubilalis* cadherin-like peptide has predicted molecular weight (192 kDa) similar to *B. mori* and *M. sexta* homologs (Compute pI/Mw program; Bjellqvist et al., 1993). Cadherins are glycoproteins involved in calcium-dependent cell–cell adhesion (Takeichi, 1990). There are 13 predicted *O. nubilalis* cadherin peptide *N*-glycosylation sites (NP[S or T]P; Gavel and von Heijne, 1990; Fig. 1). Difference between polyacrylamide mobility (220 kDa; Hua et al., 2001) and estimated 192 kDa may be due to posttranslational modifications. Predicted isoelectric point (pI) of the *O. nubilalis* cadherin peptide (4.23; Compute pI/Mw program; Bjellqvist et al., 1993) indicates solubility in alkaline midgut environments,

Fig. 1. *O. nubilalis* 1717 residue long cadherin-like peptide sequence (AY612336) with a predicted 21 amino acid signal peptide (SIG), 11 cadherin repeats (CR1 to CR11), 13 extracellular glycosylation sites, membrane proximal region (MPR), transmembrane region (TMR), and cytoplasmic domain (CPD). Putative glycosylation sites are underscored by triangles in diagram and sequence. Two potential *O. nubilalis*, *B. thuringiensis* Cry1A toxin binding regions (TBR1 and TBR2) with homology to those proposed by Gomez et al. (2002a, b, 2003) are indicated.

Fig. 2. Parsimony-based phylogeny of 12 lepidopteran cadherin peptides generated from a 3048 amino acid consensus alignment. Level of bootstrap support is indicated at each node ($N = 1000$). Branch termini labeled with GenBank accession nos. and respective species indicated as follows, Bm (*B. mori*), Ms (*M. sexta*), Cs (*Chilo suppressalis*), On (*O. nubilalis*), Pg (*P. gossypiella*), Ld (*Lymantria dispar*), Hv (*H. virescens*), and Ha (*H. armigera*). *Drosophila* cadherin Cad99C served as outgroup to root the tree.

which is congruent with required extracellular domain function.

3.2. Polymorphism within putative cadherin Cry1Ab toxin binding domains

Two putative *O. nubilalis* cadherin peptide Cry1A toxin-binding regions [861]DIEIEIIDTNN[871] and [1328]IPLQTSILVVTV[1339] were identified (Fig. 1) that were identical to 7 of 11 homologous *M. sexta* Bt-R$_1$ TBR1 residues ([865]NITIHITDTNN[875]; Gomez et al., 2001) and 9 of 12 homologous *M. sexta* Bt-R$_1$ TBR2 residues, [1331]IPLPASILTVTV[1342] (Gomez et al., 2003). A cadherin alignment of amino acid sequence (Fig. 3) indicates five TBR1 (I[868], I[870], D[872], N[874], and N[875]) and two TBR2 amino acids are fixed between all species (L[1339] and T[1341]; with respect to *M. sexta* Bt-R$_1$). Interspecies comparisons reveal little conservation of the cadherin toxin-binding region. Variability in cadherin sequences may allow flexibility of Cry1Ab toxin binding to midgut receptors or a generalized protein–protein interaction in Cry1A toxin mode of action.

Intraspecific DNA sequence alignment detected variation among cloned OnTBR1 (Fig. 4) and OnTBR2 (Fig. 5) cadherin gene PCR products, containing 214–232 and 222–232 nt introns, respectively. No insertions or deletions were observed in coding sequence (exons). In addition to intron length variation, 31 OnTBR1 SNPs were observed ($\pi = 0.02707$ (Nei, 1987); $\theta = 0.02809$ (Tajima, 1996)); 4 of 8 of these were synonymous changes in exons (Fig. 4). Thirty-one OnTBR2 SNPs were identified ($\pi = 0.02410$; $\theta = 0.02490$); 13 of 17 of these were synonymous

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          819                                                                     900
AY612336On  EAIDADEPWRFYLYYTVIASDECSLENHTECPPDPNYFEVPGDIEIEIIDTNNKVPEPLTEKFNTTVYVWENATSGDEVVQL
Onalt       --------------S-----------------S----I--------------------N--------------
AY094541Ms  GAIDADTPPRFHLYYTVVASDRCSTEDPADCPPDPTYWETEGNITIHITDTNNKVPQAETTKFDTVVYIYENATHLDEVVTL
AY118272Cs  EAIDADVPPRYHLYYQVIASDQCLED***ECPPDPMHFNTTGYIQIEILDTNNKVPRGTLGPIQVGGAHQREITQRLPVVQL
AF317621Ld  SAIDADTPPRYNLYYTIIASDQCYAENVEDCPPDPTYWETEGLISIYIIDTNNKIPQAEIELFSTTVYVWENATSGTEIETI
AY198374Pg  GAIDADIPPRWHLNYTVIASDKCSEENEENCPPDPVFWDTLGDNVINIVDINNKVPAADLSRFNETVYIYENAPDFTNVVKI
AB026260Bm  GAIDADVPRRYNLYYTVVATDRCYAEDPDDCPPDPTYWETPGQVVIQIIDTNNKIPQPETDQFKAVVYIYEDAVSGDEVVKV
AF519180Ha  QAIDADEPPRQNLYYTVIASDKCDLLTVTECPPDPTYFETPGEITIHITDTNNKVPQVEDDKFEATVYIYEGADDGEHVVQI
AF367362Hv  QAIDADEPPRQHLYYTVVASDKCDLLSVDVCPPDPNYFNTPGDITIHITDTNNRVPRVEEDKFEEIVYIYEGAEDGEHVVQL
(A)

          1287                                               1340
AY612336On  EGLFVLDQSRNVISLAQELDREVATSYTLHIAASNSPDATGIPLQTSILVVTVN
Onalt       ---------G-------------T--F--------P-------------I-
AY094541Ms  EGHFGLDPVRNRLFLKKELIREQSASHTLQVAASNSPD*GGIPLPASILTVTVT
AY118272Cs  GGYFQLDPVRNRLTLARALDQEQNRFHSIVVAASNSPSATGTPLDGTTLTVTIN
AF317621Ld  DGHFAVNPVTNVIYLVEELDREVEETYTILVAASNSPDS*VNALPSNTLTVTVN
AY198374Pg  YEPFDLDPVTNVIFLKSELDRETTATHVVQVAASNSPTGGGIPLPGSLLTVTVT
AB026260Bm  DGHFGLDETTNVLFLVKELDRSVSETYTLTIAASNSPT*GGIAL*TSTITITVN
AF519180Ha  GEHFRVDPRTNVLTLVKPLDRSEQETHTLIIGASDTP*NPAAVLQASTLTVTVN
AF367362Hv  MGHFAVDPQSNELFLLTPLERAEQETHTLIIGASDSP*SPAAVLQASTLTVTVN
(B)
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Fig. 3. Partial cadherin peptide alignment showing conservation in proximity to putative cadherin Cry1A toxin binding regions [861]DIEIEIIDTNN[871] (panel A) and [1328]IPLQTSILVVT[I/V] N[1340] (panel B) across lepidopteran lineages. Species abbreviation and GenBank accession nos. for aligned peptides are as in Fig. 2. Amino acid residues conserved with *O. nubilalis* (On) cadherin (AY612336) are highlighted. Alternate *O. nubilalis* amino acid residues (Onalt) are shown in a consensus of all sequenced alleles (Figs. 3 and 4): (A) Homologous lepidopteran amino acid sequence spanned by PCR product OnTBR1. (B) Homologous lepidopteran amino acid sequence spanned by PCR product OnTBR2-s.

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                      Primer OnCadTBR1-F
                      1>>>>>>>>>>>>>>>>>>>  E  A  I  D  A  D  E  P  W  R  F  Y  L  Y  Y  ST V70
            AY627288  CAAATTGAGGTGGATGCGAACGAGGCGATCGATGCAGACGAACCCTGGCGCTTCTACTTGTACTACTCCG
            AY627289  ----------------------------------------------------------------A--
            AY627290  ----------------------------------------------------------------A---
            AY627291  ----------------------------------------------------------------A---
            AY627292  ----------------------------------------------------------------A---
            AY627293  ----------------------------------------------------------------T---
            AY627294  ----------------------------------------------------------------A---
            AY627295  ----------------------------------------------------------------A---


                      71  I  A  S  D  E  C  S  L  E  N  R  T  E  C  P  P  D  PS N  Y  F  E  IV140
            AY627288  TCATCGCCAGCGACGAGTGCTCCCTGGAAAACCGCACGGAATGTCCTCCAGATCCCAACTACTTCGAAAT
            AY627289  -------C-----------------------A----------------C-------------A-
            AY627290  -------C-----------------------G----------------C-------------A-
            AY627291  -------T-----------------------A----------------T-------------G-
            AY627292  -------C-----------------------G----------------T-------------G-
            AY627293  -------T-----------------------A----------------T-------------G-
            AY627294  -------T-----------------------G----------------T-------------G-
            AY627295  -------T-----------------------A----------------T-------------G-
                                                                  NdeII         Tsp509I


                      141 P  G  D  ^^^^^^^^^^^^^^^^^^^^^^^^^^^^ Intron ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^210
            AY627288  TCCAGGCGATGTGAGTAATGAATATTACTGTCTGAAGCCTTTAAGGTTGTACGCTGTGTCATCATCTACT
            AY627289  -------------------G------A-------A----------GT----C---------------T
            AY627290  -------------------G------A-------A----------GT----C---------------T
            AY627291  -------------------T------G-------T----------TT----G---------------C
            AY627292  -------------------G------G-------T----------GG----G---------------C
            AY627293  -------------------G------G-------T----------GG----G---------------C
            AY627294  -------------------T------G-------T----------GT----G---------------C
            AY627295  -------------------T------G-------T----------TT----G---------------C


                      211^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^ Intron ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^280
            AY627288  GTACCTAAACCATTTGTGTGTAGTTATTTGTTGTGCTAAT*****GTTATTTCGAAAAATATGTTTATTT
            AY627289  --------------------TG-------T--G-AA--T--G------T
            AY627290  --------------------A---------TG-------*****-----T--G-AA--T--G------T
            AY627291  --------------------A---------TG-------*****-----C--*-TG--A--T******A
            AY627292  --------------------A---------TG-------ATTAT-----T--*-TG--T--T******A
            AY627293  --------------------A---------TG-------ATTAT-----T--*-TG--T--T******A
            AY627294  --------------------AT--------TG-------*****-----T--*-TG--T--T------A
            AY627295  --------------------A---------TG-------*****-----T--*-TG--A--T******A
                                                                                  TaqI
                      281^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^ Intron ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^350
            AY627288  -GAA-**ATTATAAGGGTTCCTTGTCAGAACTACGGAACCCTAAAAACCTGCTGTAATCTAATAAAAATA
            AY627289  -GAA-**ATTATAA-------------------G---------------C------------A----
            AY627290  -GAA-**ATTATAA-------------------G---------------C------------A----
            AY627291  ***T-****A***G-------------------G---------------A------------T----
            AY627292  ***T-********A-------------------G---------------C------------A----
            AY627293  ***T-********A-------------------G---------------C------------A----
            AY627294  -AGT-TCACAATAA-------------------A---------------C------------T----
            AY627295  ***T-********A-------------------G---------------A------------A----


                      351^^^^^^^^^^^^^^^ Intron ^^^^^^^^^^^^^^^^^^ I  E  I  E  I  I  D  T  N  N  K  420
            AY627288  ATAAATGCTGCAACCGTTTCGCCTTTAACTTCGACAGATCGAAATAGAAATCATCGACACAAACAACAAA
            AY627289  ---A-------------------T--------------C-----------------------------
            AY627290  --A--------------------T--------------C-----------------------------
            AY627291  ---A-------------------T--------------C-----------------------------
            AY627292  --A--------------------T--------------T-----------------------------
            AY627293  ---C-------------------T--------------C-----------------------------
            AY627294  ---A-------------------A--------------C-----------------------------
            AY627295  --A--------------------T--------------C-----------------------------


                      421V  P  E  P  L  T  E  K  F  N  T  T  V  YN V  W  E  N  A  T  S  G  D  E  490
            AY627288  GTGCCTGAGCCGCTCACTGAGAAGTTCAACACGACGGTGTACGTCTGGGAGAATGCCACTATCGGCGACG
            AY627289  ----------------------------------------T----T---------------------
            AY627290  ----------------------------------------T----T---------------------
            AY627291  ----------------------------------------A----C---------------------
            AY627292  ----------------------------------------A----T---------------------
            AY627293  ----------------------------------------A----C---------------------
            AY627294  ----------------------------------------A----T---------------------
            AY627295  ----------------------------------------A----T---------------------
                                                                      RsaI

                                   Primer OnCadTBR1-R
                      491  V  V  Q  L<<<<<<<<<<<<<<<<<<<<<<<<<523
            AY627288  AGGTGGTCCAGCTGTACTCCCACGACCGTGACA
            AY627289  -------------------------------
            AY627290  -------------------------------
            AY627291  -------------------------------
            AY627292  -------------------------------
            AY627293  -------------------------------
            AY627294  -------------------------------
            AY627295  -------------------------------
```
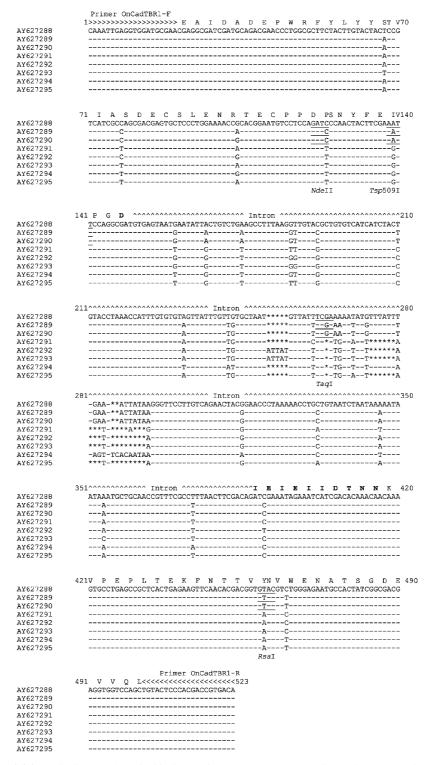
Fig. 4. Alignment of *O. nubilalis* cadherin Cry1A toxin binding region 1 (OnTBR1) genomic DNA sequence AY627288–AY627295. Primer annealing sites are indicated by arrows showing direction. Identical nucleotides (−) and deletions (*) are indicated with respect to uppermost reference sequence. Introns are overwritten (ˆ) and amino acids encoded in exons are labeled using standard 21-symbol code. Polymorphic *Nde*II, *Rsa*I, *Taq*I, and *Tsp*509I restriction endonuclease sites are underlined and labeled appropriately.

changes in exons (Fig. 5). Eight SNPs were identified within restriction endonuclease recognition sites. Four polymorphic restriction sites (*Nde*II, *Rsa*I, *Taq*I, and *Tsp*509AI) were identified from OnTBR1 (Fig. 4); and four sites (*Cfo*I, *Hae*III, *Hpy*CHVIII and *Msp*I) were identified from OnTBR2 (Fig. 5). Subsequent
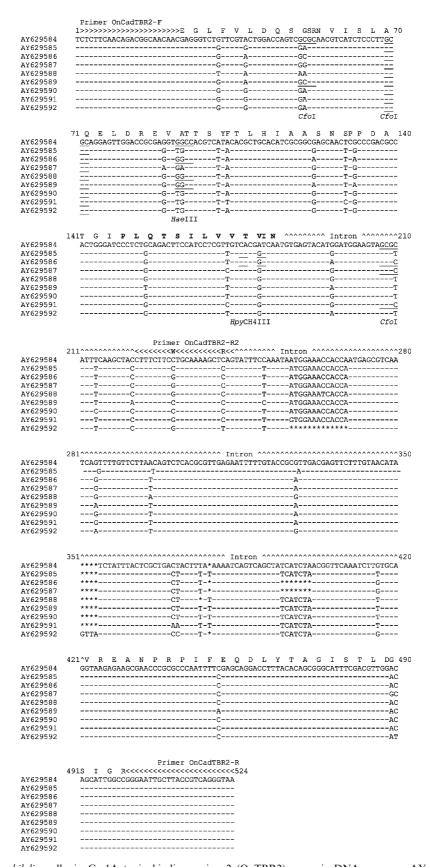
```
                 Primer OnCadTBR2-F
                 1>>>>>>>>>>>>>>>>>>>>>>>E  G  L  F  V  L  D  Q  S  GSRN  V  I  S  L  A 70
      AY629584   TCTCTTCAACAGACGGCAACAACGAGGGTCTGTTCGTACTGGACCAGTCGCGCAACGTCATCTCCCTTGC
      AY629585   --------------------------------G-----G----------GA-----------------GC
      AY629586   --------------------------------G-----A----------GC-----------------GC
      AY629587   --------------------------------G-----G----------GG-----------------GC
      AY629588   --------------------------------T-----A----------AA-----------------GC
      AY629589   --------------------------------G-----A----------GC-----------------GC
      AY629590   --------------------------------G-----G----------GA-----------------GC
      AY629591   --------------------------------G-----G----------GA-----------------GC
      AY629592   --------------------------------G-----G----------GA-----------------GC
                                                                 CfoI              CfoI

                 71 Q  E  L  D  R  E  V  ATT  S  YF T  L  H  I  A  A  S  N  SP P  D  A 140
      AY629584   GCAGGAGTTGGACCGCGAGGTGGCCACGTCATACACGCTGCACATCGCGGCGAGCAACTCGCCCGACGCC
      AY629585   -G-------------------G--TG------T-A-------------------G------T-G--------
      AY629586   -------------------G--GG-------T-A-------------------A------T-A---------
      AY629587   -------------------A--GA------T-A-------------------G------T-G---------
      AY629588   -------------------G--GG-------T-A-------------------G------T-G---------
      AY629589   -------------------G--GG-------T-A-------------------A------T-A---------
      AY629590   -G-------------------G--TG------T-A-------------------G------T-G---------
      AY629591   -------------------G--TG-------T-T-------------------G------C-G---------
      AY629592   -G-------------------G--TG------T-A-------------------G------T-G---------
                                         HaeIII

                 141T  G  I  P  L  Q  T  S  I  L  V  V  T  VI N  ^^^^^^^^^ Intron ^^^^^^^^210
      AY629584   ACTGGGATCCCTCTGCAGACTTCCATCCTCGTTGTCACGATCAATGTGAGTACATGGATGGAAGTAGCGC
      AY629585   --------------G----------------T-----G-----------------G------------T
      AY629586   --------------G----------------T---G-----------------A------------C
      AY629587   --------------G----------------C-----G-----------------G----------C
      AY629588   --------------G----------------T-----G-----------------G------------T
      AY629589   --------------T----------------T-----G-----------------A------------T
      AY629590   --------------G----------------T-----G-----------------G------------T
      AY629591   --------------G----------------C-----G-----------------G----------C
      AY629592   --------------G----------------T-----G-----------------A------------T
                                 HpyCH4III                                 CfoI

                 Primer OnCadTBR2-R2
                 211^^^^^^^^^^^<<<<<<<W<<<<<<<<<<<R<<^^^^^^^^^ Intron ^^^^^^^^^^^^^^^^^^^280
      AY629584   ATTTCAAGCTACCTTTCTTCCTGCAAAAGCTCAGTATTTCCAAATAATGGAAACCACCAATGAGCGTCAA
      AY629585   ---T-------C--------C----------C--------T-----ATCGAAACCACCA-----------
      AY629586   ---T-------C--------G----------T--------C-----ATGGAAACCACCA-----------
      AY629587   ---T-------C--------C----------C--------T-----ATGGAAACCACCA-----------
      AY629588   ---T-------C--------G----------C--------T-----ATGGAAATCACCA-----------
      AY629589   ---T-------A--------C----------C--------C-----ATGGAAACCACCA-----------
      AY629590   ---C-------C--------C----------C--------T-----ATGGAAACCACCA-----------
      AY629591   ---T-------C--------G----------C--------T-----GTGGAAACCACCA-----------
      AY629592   ---T-------C--------G----------T--------T-----*************-----------

                 281^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^ Intron ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^350
      AY629584   TCAGTTTTGTTCTTAACAGTCTCACGCGTTGAGAATTTTTGTACCGCGTTGACGAGTTCTTTGTAACATA
      AY629585   ---G----------T-------------------------------A---------------------
      AY629586   ---G----------T-------------------------------A---------------------
      AY629587   ---G----------T-------------------------------A---------------------
      AY629588   ---G----------A-------------------------------G---------------------
      AY629589   ---A----------T-------------------------------A---------------------
      AY629590   ---G----------T-------------------------------A---------------------
      AY629591   ---G----------T-------------------------------A---------------------
      AY629592   ---A----------T-------------------------------G---------------------

                 351^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^ Intron ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^420
      AY629584   ****TCTATTTACTCGCTGACTACTTTA*AAAATCAGTCAGCTATCATCTAACGGTTCAAATCTTGTGCA
      AY629585   ****---------------CT----T-T---------------TCATCTA--------------T----
      AY629586   ****---------------CT----T-*---------------******--------------G----
      AY629587   ****---------------CT----T-*---------------******--------------G----
      AY629588   ****---------------CT----*-T---------------TCATCTA--------------T----
      AY629589   ****---------------CT----T-T---------------TCATCTA--------------T----
      AY629590   ****---------------CT----T-T---------------TCATCTA--------------T----
      AY629591   ****---------------AA----T-T---------------TCATCTA--------------T----
      AY629592   GTTA---------------CC----T-*---------------TCATCTA--------------G----

                 421^V  R  E  A  N  P  R  P  I  F  E  Q  D  L  Y  T  A  G  I  S  T  L  DG 490
      AY629584   GGTAAGAGAAGCGAACCCGCGCCCAATTTTCGAGCAGGACCTTTACACAGCGGGCATTTCGACGTTGGAC
      AY629585   -----------------------------C--------------------------------------AC
      AY629586   -----------------------------C--------------------------------------AC
      AY629587   -----------------------------C--------------------------------------GC
      AY629588   -----------------------------C--------------------------------------AC
      AY629589   -----------------------------A--------------------------------------AC
      AY629590   -----------------------------C--------------------------------------AC
      AY629591   -----------------------------C--------------------------------------AC
      AY629592   -----------------------------C--------------------------------------AT

                 Primer OnCadTBR2-R
                 491S  I  G  R<<<<<<<<<<<<<<<<<<<<<<<<<<524
      AY629584   AGCATTGGCCGGGAATTGCTTACCGTCAGGGTAA
      AY629585   ----------------------------------
      AY629586   ----------------------------------
      AY629587   ----------------------------------
      AY629588   ----------------------------------
      AY629589   ----------------------------------
      AY629590   ----------------------------------
      AY629591   ----------------------------------
      AY629592   ----------------------------------
```

Fig. 5. Alignment of *O. nubilalis* cadherin Cry1A toxin binding region 2 (OnTBR2) genomic DNA sequence AY629584–AY629592. Primer annealing sites are indicated by arrows showing direction. Region bound by primers OnCadTBR2-F and OnCadTBR2-R encompasses the PCR product OnTBR2. Identical nucleotides (-) and deletions (*) are with respect to uppermost reference sequence. Introns are overwritten (ˆ) and amino acids encoded in exons are labeled using standard 21-symbol code. Polymorphic *Cfo*I, *Hae*III, *Hpy*CH4III, and *Msp*I restriction endonuclease sites are underlined and labeled appropriately within the region OnTBR2-s amplified with primers OnCadTBR2-F and OnCadTBR2-R2.

PCR-RFLP assays used PCR product OnTBR2-s for [1328]IPLQTSILVVTV[1339] region SNP detection. One SNP within an *Hpy*CHVIII recognition site represents a nonsynonymous peptide change ([1328]IPLQTSILVV-T[I/V] N[1340]). Alternate amino acids (I or V) both have short chain aliphatic side groups and may not affect regional charge or peptide structure. Remaining PCR-RFLP indicate nonsynonymous changes of cadherin alleles outside [861]DIEIEIIDTNN[871] and [1328]IPLQT-SILVVT[I/V] N[1340] (Figs. 4 and 5).

The nucleotide substitutions in the cadherin gene and PCR-RFLP assays provide a means for genotyping *O. nubilalis*, mapping of resistance traits, and characterizing populations. Three cadherin alleles (r1, r2, and r3) in Cry1Ac resistant *P. gossypiella* strain AZP-R differed by block deletion or truncation (Morin et al., 2003), and shared no fixed peptide sequence differences compared to alleles from a susceptible line. Resistant allele r2 had a truncation deletion that eliminated homologous *P. gossypiella* TBR1 ([862]DNVINIVDINNK[873]) and TBR2 ([1341]PLPGSLLTVTVT[1352]); and allele r1 had a nonsynonymous change directly flanking TBR1 (R[861]) compared to of susceptible and r3 alleles (G[861]). Morin et al. (2003) also established a correlation between cadherin resistance alleles and increased *P. gossypiella* larval weight among individuals from a Texas field sample (TX-01) reared on diet containing 1 μg Cry1Ac/ml. Recessive Bt resistant alleles were independently selected for in *P. gossypiella* strain APHIS-98R (Tabashnik et al., 2004), suggesting utility of fielding surveillance to monitor the development of resistance.

3.3. Mendelian inheritance and cadherin allele frequencies

Mendelian inheritance of *O. nubilalis* cadherin alleles was established within F_2 pedigrees, Ped31 and Ped62. Genotype ratio expected among F_2 progeny was determined assuming Mendelian segregation of parental alleles. The observed genotype did not significantly deviate from expected ratios in either pedigree for assays in which parentals were polymorphic (χ^2 test; Table 1). Adherence of F_2 progeny to expected Mendelian ratios suggests null alleles are not present. PCR-RFLP assays are therefore appropriate as markers for linkage mapping or population genetic studies (Pemberton et al., 1995).

Molecular screening may detect geographic regions of increased insecticide resistance levels and are increasingly being incorporated into resistance diagnostics (Brogdon and McAllister, 1998). Molecular assays have been developed to detect resistance genes in insect populations (Steichen and Ffrench-Constant, 1994; Field et al., 1996), and screening known resistance alleles in *P. gossypiella* may show promise for population surveillance (Morin et al., 2003; Tabashnik et al., 2004). In a preliminary surveillance experiment we used PCR-RFLP to estimate frequencies of *O. nubilalis* cadherin alleles (SNP haplotypes) in a population of susceptible adults collected near Mead, Nebraska. Three to six alleles were observed per PCR-RFLP assay (OnTBR1 *Rsa*I, *Taq*I, and *Tsp*509I, and OnTBR2-s *Cfo*I and *Hae*III with *Hpy*CH4 III) and 21 unique allelic combinations (genotypes) were observed. Frequency of cadherin alleles were defined from individual restriction assays and ranged from 0.012 to 0.455 (Table 2).

One or more genes may mediate lepidopteran resistance to Cry1A toxins. Aminopeptidase was implicated as a major midgut receptor (Knight et al., 1994; Gill et al., 1995), followed by cadherin (Vadlamudi et al., 1995). Screening *P. xylostella* for survival on Cry1A and Cry1F toxins suggested cross resistance was imparted by a single gene (Tabashnik et al., 1997). This was corroborated when a major autosomal locus inherited as a recessive trait was identified (Heckel et al., 1999). Cadherin was implicated as a resistance factor by phenotypic association in *P. gossypiella* (Morin et al., 2003) and transposon knockout in *H. virescens* (Gahan et al., 2001). For *Spodoptera litura*, transcript knockdown by RNA interference (RNAi) showed the role of aminopeptidase in Bt resistance (Rajagopal et al., 2002). Inheritance of resistance in an *O. nubilalis* strain selected for survival on Dipel® Bt formulations was autosomal and incompletely dominant (Huang et al., 1999). Additive gene action was predicted to mediate Bt resistance in *H. zea* (Burd et al., 2003); and two copies of cadherin resistance alleles (r1, r2, or r3) were required for resistance in *P. gossypiella* (Morin et al., 2003).

Table 1
Mendelian inheritance of cadherin alleles in *O. nubilalis* pedigrees Ped31 and Ped62

Pedigree	Parental genotype	Observed cadherin PCR-RFLP genotype ratios among F_2 progeny	Expected F_2 ratio	χ^2	*p*-Value (df)
Ped31	♂R15♀R16	TBR1; R11(13/39): R16(12/39): R15(7/39) R56(6/39): R55(0/39): R66(1/39)	4:4:4:2:1:1	5.92	0.205 (4)
	♂C12♀C11	TBR2; C11(26/39): C12(14/39): C22(4/39)	9:6:1	1.07	0.586 (2)
Ped62	♂T22♀T11	TBR1; T11(10/44): T12(26/44): T22(8/44)	1:2:1	1.64	0.441 (2)
	♂H33♀H34	TBR2; H33(26/44): H34(14/44): H44(4/44)	9:6:1	1.01	0.604 (2)

Observed genotypic ration among F_2 offspring was compared to Mendelian expected F_2 ratio based on parental genotypes (R15 genotype is R1 and R5 heterozygote; see PCR-RFLP alleles on Table 1). Significance of observed allele frequency departures from expected tested by Chi-square (χ^2).

Table 2

Mead, Nebraska *O. nubilalis* frequency of cadherin PCR-RFLP alleles (Table 2)

PCR-RFLP	RE Fragments	AA	A_O
OnTBR1 *RsaI* Alleles			
R1	241, 130, 61, 45, 21, 18	Y^{886}	0.341
R2	270, 151, 61, 18	N^{866}	0.012
R3	275, 151, 61, 18	N^{866}	0.085
R4	287, 151, 61, 18	N^{866}	0.012
R5	241, 151, 61, 45, 18	N^{866}	0.244
R6	250, 151, 61, 45, 18	N^{866}	0.305
OnTBR1 *TaqI* Alleles			
T1	241, 119, 105, 29, …	Intron	0.450
T2	224, 119, 105, 29, …	Intron	0.431
T3	123, 119, 117, 105, …	Intron	0.060
T4	229, 119, 105, 29, …	Intron	0.060
OnTBR1 *Tsp509I* Alleles			
P1	Uncut	V^{858}	0.085
P2	365, 158	V^{858}	0.220
P3	251, 158, 114	V^{858}	0.384
P4	239, 148, 139	I^{858}	0.311
OnTBR2-s *CfoI* Alleles			
C1	174, 70	G^{1296}/S^{1296}	0.455
C2	138, 51, 36, 19	R^{1296}	0.141
C3	174, 51, 19	R^{1296}	0.327
C4	138, 70, 36	G^{1296}/S^{1296}	0.077
OnTBR2-s *HaeIII* & *HpyCH4III* Alleles			
H1	151, 93	$A^{1310} I^{1339}$	0.330
H2	177, 67	$T^{1310} V^{1339}$	0.304
H3	93, 84, 67	$A^{1310} V^{1339}$	0.357

Restriction enzyme (RE) fragment sizes in base pair, and alternate amino acid (AA) detected by PCR-RFLP given for each allele. Observed allele frequency (A_O) were calculated.

To manage resistance effectively, it is necessary to understand the genetic mechanisms of resistance (Gould and Tabasnnik, 1998; Caprio et al., 2000) and monitor insect pests of transgenic-insecticidal cultivars for changes in their tolerances of Bt toxins (Gould et al., 1997; Andow and Alstad, 1998). To do this, it is necessary to track the evolutionary changes of resistance alleles in pest populations. The full-length mRNA, partial-genome sequence, and molecular markers developed in this study have downstream applications that will aid in the determination of cadherin involvement in potential *O. nubilalis* resistance to Cry1Ab toxin. These markers are appropriate for incorporation into linkage mapping studies. Laboratory colonies of *O. nubilalis* that exhibit moderate resistance to Cry1Ab toxin in artificial diet (Bolin et al., 1999; Huang et al., 1999; Chaufaux et al., 2001), and diagnostic bioassays can discriminate phenotypes (Marcon et al., 2000). OnTBR1 and OnTBR2 PCR-RFLP markers show Mendelian inheritance (Table 1), which is required for genetic mapping procedures (Pemberton et al., 1995). F_2 pedigrees in conjunction with the bioassays can be used to determine if the segregation of the moderate *O. nubilalis* resistance phenotype is associated with cadherin allele segregation using OnTBR1 or OnTBR2 PCR-RFLP assays. A similar protocol was used to associate cadherin alleles r1, r2, and r3 with Cry1Ac resistance in the *P. gossypiella* AZP-R strain and field collections (Morin et al., 2003). Association of cadherin alleles with distinct phenotypic traits is a prerequisite for effective field surveillance, but marker polymorphism determines how effectively alleles are differentiated. Our cadherin loci will also be incorporated into linkage maps of other marker types (AFLP and microsatellites) to better examine cause and effect between resistant phenotypes and cadherin loci. This will help pinpoint the location of putative resistant loci and ask if their location is in the vicinity of our cadherin maker loci.

Polymorphisms among *O. nubilalis* cadherin alleles at Mead, Nebraska for OnTBR1 and OnTBR2 indicates that native genetic variation is present. The native genetic variation for cadherin loci can be incorporated into resistance monitoring or surveillance programs, pending allelic association with potential field resistance traits via bioassays. Studies are currently underway with *O. nubilalis* to map the genomic position of the cadherin locus. We will use the information obtained not only to better understand inheritance of Cry1Ab toxin resistance, but also examine the appropriateness of allelic association with phenotypic traits for potential incorporation into field monitoring or surveillance programs.

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